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14. ABSTRACT

The purpose of this research is to determine the extent to which exfoliated epithelial cells present in breast milk can be used to assess a woman's individual risk of developing breast cancer and to detect early signs of breast cancer. To accomplish this goal we have collected breast milk samples from 250 lactating women who either have had a breast biopsy or are scheduled for a breast biopsy. We have isolated the epithelial cells and are determining the DNA promoter methylation patterns of several tumor-suppressor genes that are frequently methylated in breast cancer. Tasks 1 through 5 have been completed and we are making significant progress on Tasks 6 and 7. We completed methylation analyses of three genes (RASSF1, SFRP1 and GSTP1) on all samples and will be presenting the results of this research at the annual meeting of the American Association of Cancer Research. We requested and received a one year extension during which we will complete the methylation and statistical analyses on the remaining six genes.

15. SUBJECT TERMS
Breast milk; recruitment; promoter hypermethylation; RASSF1, GSTP1, SFRP1

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Introduction

The purpose of this research is to determine the extent to which the promoter methylation pattern in exfoliated epithelial cells present in breast milk can serve as a reliable means of assessing an individual woman's risk of developing breast cancer. We are collecting breast milk samples from 250 lactating women who either are scheduled for a breast biopsy or have had a breast biopsy in the past. Women provide milk from both the biopsied and non-biopsied breast as well as a copy of their biopsy report. They also complete a health and reproductive history questionnaire. From each milk sample we isolate the epithelial cells from the total cell population and determine the methylation pattern of selected genes using pyrosequencing of bisulfite-modified DNA. Based on published statistics, we expect that roughly 10% or 25 milk samples will come from women with breast cancer. This design provides a unique opportunity to assess the development of cancer-like methylation patterns in premenopausal women who either have breast cancer or a benign lesion and to compare the cells from the diseased breast with those from the healthy breast. We also will compare the methylation profiles we obtain from this At-Risk population (At-Risk because they have needed a breast biopsy) with the methylation profiles we obtained from women at Average-Risk (Wong *et al.*, 2010

Body

As noted in the previous annual report, during the first year of the project we completed Tasks 1 – 4 and made significant progress on Tasks 5 and 6. During the second year of this study we have completed subject recruitment and sample collection (Task 5). We have been extremely successful in subject recruitment. A detailed review of our success in recruitment to date is provided in ***Appendix 1*** (a PowerPoint slideshow on Subject Recruitment and Biopsy Types and Diagnoses). We have exceeded our goal of collecting breast milk samples from 250 women, we currently have received milk samples from 252 women and an additional 24 milk collection have been sent to women. We asked for and received a one-year no additional-cost extension to complete this research project and we therefore are continuing to accept breast milk samples from eligible women.

To date we have obtained the biopsy reports from 200 of the 252 women who sent breast milk samples. We continue to assist women in obtaining their biopsy reports. This assistance takes various forms including contacting subjects, physicians, and clinics both by phone and letter. The PI will continue to seek these biopsy reports and we expect to obtain reports from nearly all the women.

Of the 276 lactating women who were sent milk boxes, 18 were scheduled to have a biopsy. The vast majority of women enrolled in the study had previous biopsies (244) and a small number (14) neither had a biopsy nor were schedule to have a biopsy but were considered at increased risk for breast cancer and therefore were sent a box. Of the 200 lactating women from whom we have received biopsy reports, 14 have been diagnosed with cancer. The remainder has benign lesions as detailed in slides 6 through 8 of Appendix 1.

All of the milk samples collected from the 252 women have been processed, that is the cell populations have been separated and the DNA has been isolated and modified with bisulfite-treatment. The bisulfite modified DNA has been aliquoted and stored at - 80°C. Frozen aliquots are thawed for methylation analysis by pyrosequencing. PCR amplification and pyrosequencing has been completed for three genes, RASSF1, SFRP1 and GSTP1 and have completed some initial analyses. As shown Figure 1, both percent methylation scores (y axis) and the percentage of women with high methylation scores (outliers) is significantly greater in the present study of women who have had biopsies (At-Risk Population) than in previous study of women at average-risk.

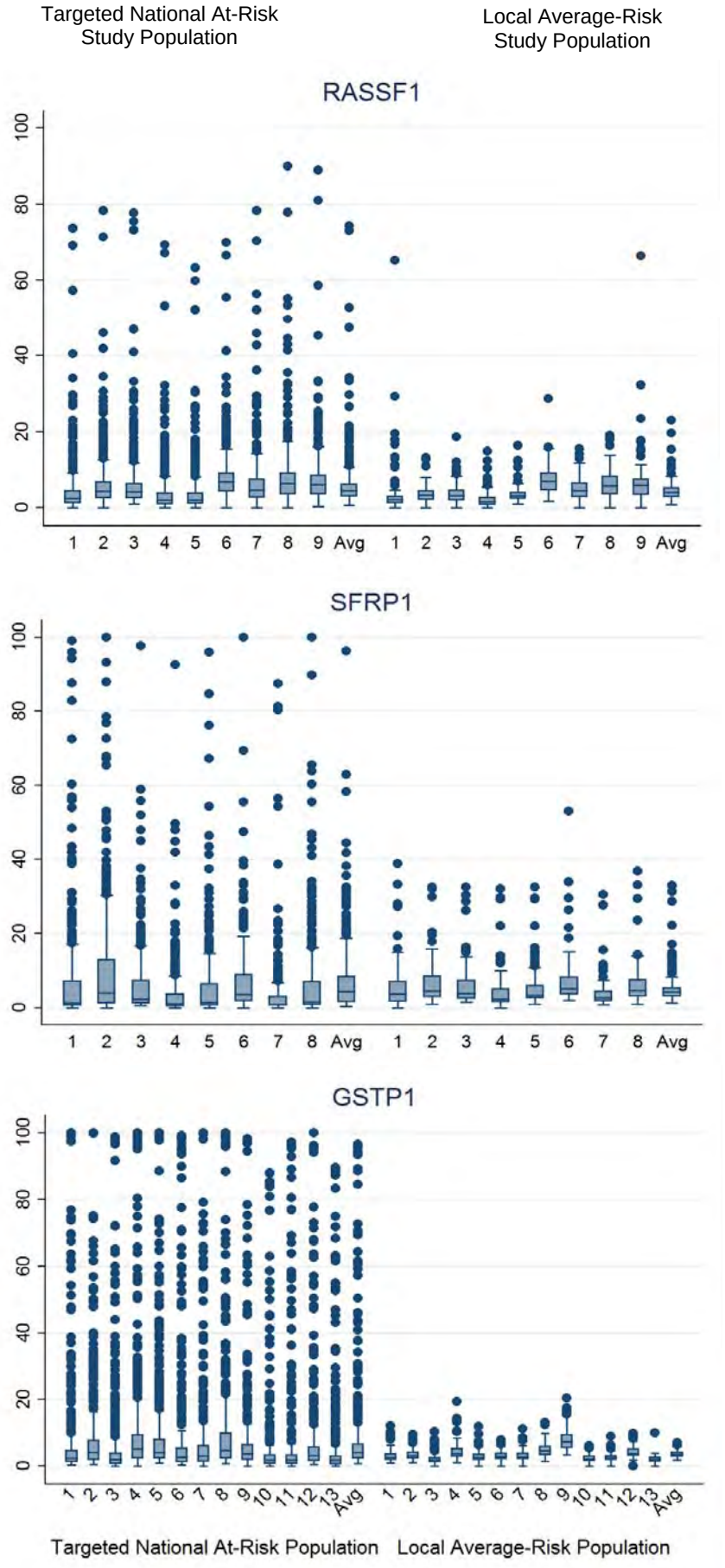

Figure 1. More frequent breast epithelial cell DNA methylation is detected in a national at-risk study group as compared to a local average-risk study group Exfoliated epithelial cells were isolated from the breast milk of two groups of women: 1) 250 lactating women recruited from across the nation to participate in a study to identify molecular biomarkers of breast cancer risk in exfoliated breast epithelial cells (left side) and 2) 102 lactating women recruited from the Amherst, MA area to participate in a study to identify age- and parity-related changes in exfoliated breast epithelial cells (right side).

Eligibility for the at-risk study required that the woman have had or be scheduled to have a breast biopsy (due to a suspicious lump *etc.*), be willing to provide a copy of her biopsy report, complete a questionnaire and donate a milk sample from each breast (if possible). In contrast, eligibility for the average-risk study required only that the woman be willing to donate a milk sample and complete a questionnaire.

DNA isolated from the epithelial cell fraction was modified by bisulfite treatment, promoter regions known to be methylated in breast cancer were amplified, and PCR products were analyzed by pyrosequencing. Y axis equals percent methylation and X axis shows the individual CpG mean methylation scores and the average overall sites for each of the two study groups. While the mean methylation scores for the three genes shown (RASSF1, SFRP1 and GSTP1) do not differ significantly between the two groups, the percent of outliers is significantly higher in the at-risk group.

Importantly, all of the women diagnosed with cancer in the At-Risk group (n = 14) were removed from the analysis (no women in the Average-Risk group were diagnosed with cancer). Therefore the comparison between the two study groups represents ***breast-cancer risk.***

As shown in Figure 2 it is necessary to separate the epithelial and non-epithelial cell fractions as the two cell populations clearly provide different information and analysis of the epithelial cells is most relevant for assessing breast-cancer risk.

As outlined in our proposal we have examined both the epithelial-enriched and epithelial-depleted cell fractions from both the biopsied and non-biopsied breasts. We have determined that the epithelial-enriched cell fraction provides unique information that is most relevant to breast cancer risk. Therefore, for the remaining six genes (CDH1, DAPK, ERα, HIN1, RARβ, and p16), we will first analyze the epithelia-enriched cell fraction of both breasts. This will allow us to complete the analyses on the enriched fraction and prepare manuscripts while we continue the analysis on the epithelial-depleted cell fractions. After completing methylation analyses in the epithelia-enriched cell fractions we will analyze the methylation patterns in the epithelial-depleted cell fractions.

We are in the initial stages of analyzing the data. As mentioned above, analysis of the exfoliated epithelial cell DNA from women with benign biopsies revealed a subset of the population with increased methylation scores predicting that they are at increased risk of developing breast cancer. We also have compared results between the biopsied and nonbiopsied breasts from both women with benign disease and cancer and will be presenting these results at the national meeting of the American Association for Cancer Research in April, 2011). Briefly, in women whose biopsy showed a non-proliferative lesion, there was no significant difference in the average epithelial DNA methylation of their biopsied breast compared to their non-biopsied breast for RASSF1 (p=0.15) and GSTP1 (p=0.92), but for SFRP1 the average methylation was significantly higher in the biopsied breast (p= 0.02). In women whose biopsy result revealed cancer, there was a significant increase in average RASSF1 methylation of cells form their biopsied breast compared to their non-biopsied breast (p=0.05).

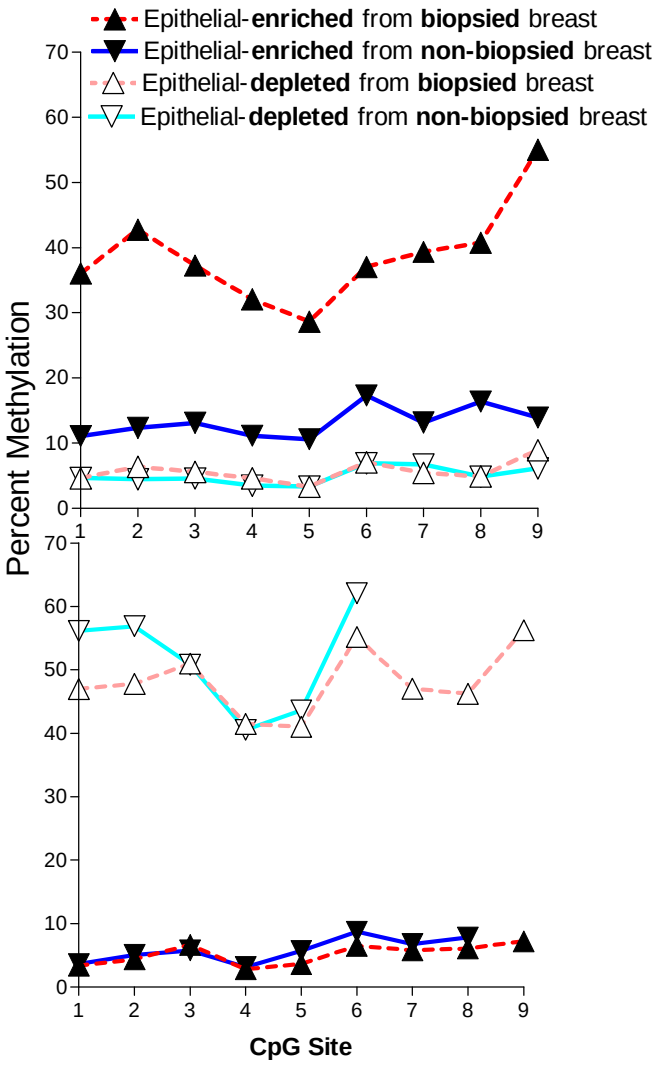

Figure 2. Separation of cell types in milk reveals different methylation profiles. RASSF1 promoter methylation (9 CpG sites) in the epithelial-enriched and -depleted cell populations isolated from **the breast milk** of a woman with a complex fibro adenoma (TOP) and a woman with an angiolipoma (BOTTOM). ***Note the difference in methylation by cell fractions and biopsy status.*** The woman with the epithelial cell lesion (fibro adenoma) shows the highest methylation in the ***epithelial-enriched*** cell fraction of the ***biopsied breast***. In contrast, the woman with a non-epithelial cell lesion shows increased methylation in the epithelial-depleted cell fraction of both breasts

Key Research Accomplishments

- Collected breast milk samples from 252 women
- Obtained biopsy reports from 200 women
- Processed milk samples from 252 women (from both right and left breasts)
- Isolated DNA from all cell samples (both epithelial and non-epithelial fractions from both breasts)
- Bisulfite-treated all DNA samples (both epithelial and non-epithelial fractions from both breasts)
- Determined promoter methylation profile for RASSF1, GSTP1 and SFRP1 in all samples
- Continuing long-term follow- up all women enrolled in this study and our previous breast milk study

Reportable Outcomes

Received one award based on the breast milk samples being collected for the present study

Avon Foundation: *Impact of Environmental Estrogens on Epigenetics: Bisphenol A in Breast Milk and Promoter Methylation of Exfoliated Breast Epithelial Cells*
The goal of this study is to determine the relationship between levels of Bisphenol A (BPA) in breast milk and promoter methylation. We are examining BPA in the breast milk we collected from the participants in the present study. IRB protocol allows the use of the milk for other studies.

Submitted a grant proposal based on the preliminary results of the present study.

Avon Foundation: "Epigenetics and Breast Cancer Risk in African American Women" (PI: Kathleen Arcaro)

Presented results from the research at the annual AACR meeting in 2010

Browne EP, Punska EC, Lenington S, Anderton DL, Arcaro KF. 2010. RASSF1A Promoter Methylation in Exfoliated Breast Cells Isolated from Breast Milk Donated by Women Who Have Had a Breast Biopsy. AACR 101st Annual Meeting, Washington D.C., April

Conclusion

During the second year of this award we have reached our recruitment goal and obtained breast milk samples from 252 women. We have processed all milk samples and completed the methylation analyses for three genes, RASSF1, GSTP1 and SFRP1. We are continuing the methylation analyses for the remaining six genes. Results based on the analysis of the first three genes clearly indicate that women at increased risk of developing breast cancer have higher methylation scores in their exfoliated epithelial cells. The extent to which breast milk can be used to assess breast cancer risk will be determined only with long-term follow-up, which we are conducting.

Recently the United States Preventative Task Force issued new guidelines for screening mammography. These new guidelines specify that for women at average risk, mammographic screening for breast cancer should begin at age 50 instead of the previously recommended age 40. Earlier mammographic screening continues to be recommended for women at high risk. In this context, it is particularly important to be able to identify women at high risk who may benefit by early initiation of mammographic screening. Our results indicate the breast milk will provide a tool for identifying lactating women at increased risk of developing breast cancer.

References

Wong, C.M., Anderton, D.L., Smith-Schneider, S., Wing, M.A., Greven, M.C. and ***Arcaro, K.F.*** 2010 Quantitative analysis of promoter methylation in exfoliated epithelial cells isolated from breast milk of healthy women *Epigenetics* 5(7): 1-11.

Appendices

1. PowerPoint slides on “Update on Subject Recruitment and Biopsy Types and Biopsy Diagnoses”

DOD Breast milk study: update on recruitment and biopsy types

March 3, 2011

Kathleen Arcaro
University of Massachusetts Amherst

1

Contact and Recruitment Numbers

Number of Women Contacted	477
Number of Women Who Responded	328
Number of Women Recruited	276
Number of Women Completed	218

2

Categories of Women Recruited

Category	Number of Women Sent Boxes	Number of Milk Samples Returned	Number of Biopsy Reports Received
Biopsy Planned	18	11	9
Previous Biopsy	244	223	191
No Biopsy	14	18	na
Total	276	252	200

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Biopsy Types

- FNA = 23
- Core Biopsy = 69
- Surgical Biopsy = 100
- Unknown = 8

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Malignant Diagnoses

- Ductal carcinoma in situ (DCIS) = 4
- Invasive ductal carcinoma (IDC) = 3
- DCIS + IDC = 4
- IDC+ invasive lobular carcinoma = 1
- "cancer" = 2

- We also have received frozen milk samples from 3 additional women who have been diagnosed with breast cancer

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Biopsy Diagnoses

American Cancer Society classifies benign diagnoses into three levels of risk

- Non-proliferative lesions – none or very small elevation of risk (fibrocystic disease (fibrosis and/or cysts) mild hyperplasia (an abnormal overgrowth of cells) , adenosis (non-sclerosing, or non-hardening of tissue) , simple fibroadenoma , phyllodes tumor (benign) , a single papilloma , fat necrosis , mastitis , duct ectasia , other benign tumors (lipoma, hamartoma, hemangioma, neurofibroma) : N=153
- Proliferative lesions without atypia – 1/1/2 – 2 x elevation of risk (usual ductal hyperplasia (without atypia) complex fibroadenoma . sclerosing adenosis , several papillomas or papillomatosis . radial scar : N=31
- Proliferative lesions with atypia – 3-5 x elevation of risk (atypical ductal hyperplasia , atypical lobular hyperplasia) *: N= 1

* We also include lobular carcinoma in situ (LCIS) in this highest risk category

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Biopsy Diagnoses Non-Proliferative Lesions

Category	N
adenoma	4
adenosis	3
adipose tissue	2
angiolipoma	1
benign	7
cyst	8
duct ectasia	1
fat necrosis	1
FCC	13
fibroadenoama	58
fibroadenosis	1

Category	N
fibrosis	14
galactocele	2
granuloma	1
harmartoma	1
Hemangioma	1
lymph node	2
lactational changes	16
lipoma	1
mastopathy	1
mastitis	4
negative	10
phyllodes tumor	1

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Biopsy Diagnoses Proliferative Lesions with and without Atypia

Without Atypia

Category	N
complex fibroadenoma	2
ductal hyperplasia	16
FCC proliferative	2
lobular hyperplasia	2
papilloma	1
radial scar	1
sclerosing adenosis	8

With Atypia

Category	N
atypical ductal hyperplasia	1

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